Table 1 Baseline characteristics of all patients (groups 1 and 2)

Characteristic	SVR ($n = 58$)			Non-SVR ($n = 20$)			p value[△]
	Group 1 ($n = 36$)	Group 2 ($n = 22$)	Combined ($n = 58$)	Group 1 ($n = 7$)	Group 2 ($n = 13$)	Combined ($n = 20$)	
Gender (male/female)	20/16	9/13	29/29	4/3	5/8	9/11	0.80[†]
Age (years)	50.0 ± 12.5*	57.3 ± 10.0	52.4 ± 12.1	55.0 ± 9.7	59.8 ± 6.4	58.1 ± 7.8	0.058[‡]
ALT (IU/l)	86.6 ± 86.6	71.2 ± 50.4	80.5 ± 74.2	52.9 ± 29.3	88.1 ± 90.1	75.8 ± 75.5	0.81[‡]
Platelet ($\times 10^4/mm^3$)	20.8 ± 6.2	19.0 ± 5.2	20.1 ± 5.8	14.7 ± 7.1	19.1 ± 4.9	17.6 ± 6.0	0.11[‡]
Fibrosis score (0–2/≥3)[§]	34/1	19/2	53/3	4/3	11/2	15/5	0.049[†]
HCV RNA (KIU/ml)	760 (2–3,100)**	340 (54–3,600)	550 (12–3,600)	1,300 (350–30,000)	1,400 (180–5,000)	1,300 (180–30,000)	0.002[‖]
IFN dose (≥80%/60–80%)[¶]	28/4	21/1	49/5	4/3	11/2	15/5	0.12[†]
Ribavirin dose (≥80%/60–80%)[¶]	27/5	17/5	44/10	4/3	5/8	9/11	0.003[†]
RVR rate (%)	87.5	54.5	74.1	33.3	46.1	42.1	0.022[†]
EVR rate (%)	100	100	100	66.7	100	89.4	0.07[†]

* Mean ± SD; ** median (range); [†] Fisher's exact probability test; [‡] Student t test; [‖] Mann–Whitney's U test; [△] p values between all SVR ($n = 58$) versus all non-SVR ($n = 20$)

Several clinical characteristics listed above were unavailable in some patients. § SVR: $n = 56$ (35 in group 1, 21 in group2), non-SVR: $n = 17$ (7 in group 1, 10 in group 2); [¶] SVR: $n = 54$ (32 in group 1, 22 in group 2)

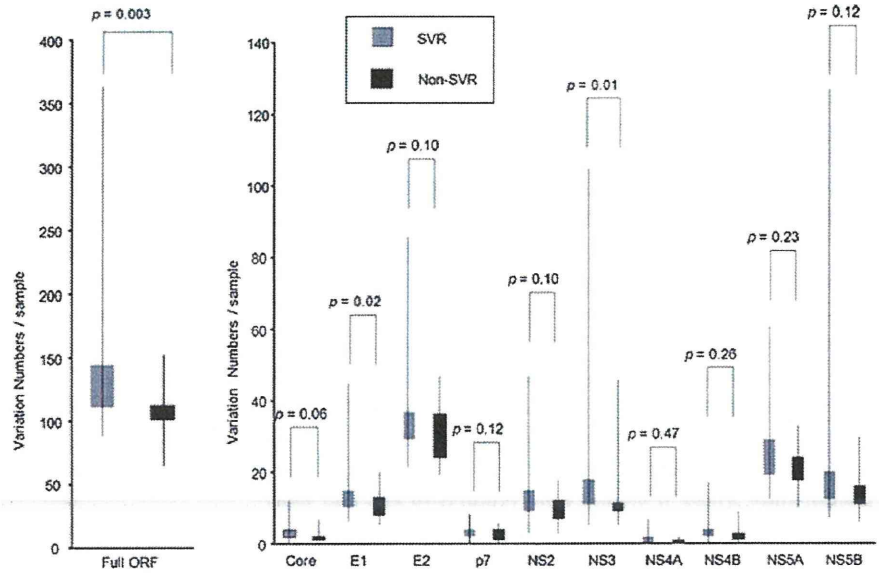

Fig. 1 Number of amino acid substitutions per sample in the sustained viral responders (SVR) and the non-sustained viral responders (non-SVR) group. The numbers of variations, relative to a population consensus, that were unique to either SVR or non-SVR patients are shown for the full ORF (*left*) and for each HCV protein (*right*)

Comparison of HCV sequence variation between the SVR and non-SVR patients at each amino acid position

Next, each amino acid position in the HCV ORF was compared to detect any differences between the SVR and non-SVR patients after determination of the consensus sequence from all 78 patients. In Fig. 2a, the final differences of the two independent studies combined are shown as dots demonstrating –log P values. As shown in the figure, amino acid usage at amino acid 110 in the core region differed strikingly between the two groups ($p = 5E{-}05$). The site was detected in group 1 ($p = 0.01$) and was validated in group 2 ($p = 0.004$) (Table 2), and the final p value became remarkably high, making the p value at this site most significantly low. Variations of aa 773 in p7, aa 2099 in the NS5A, and aa 3013 in NS5B were also shown to differ significantly between the SVR and the non-SVR patients when the two studies were combined; however, they were not confirmed by one of the studies (Table 2). Figure 2b shows the aligned sequences of amino acids 1–120 of the core region. Substitutions at aa 110 from

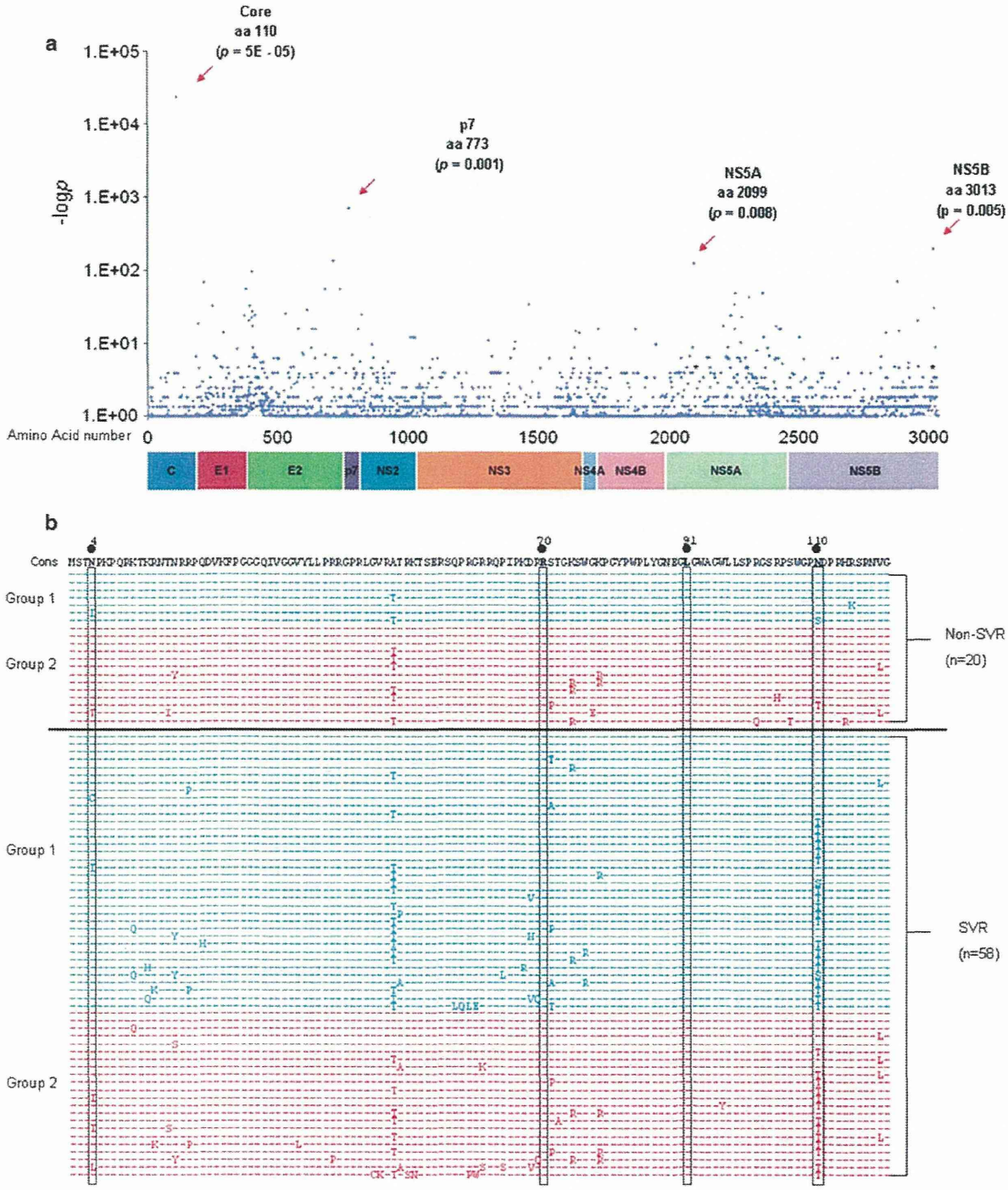

Fig. 2 **a** Different amino acid usages at each viral amino acid position between the sustained viral responders (SVR) and the non-sustained viral responders (non-SVR) patients. Amino acid variation was determined between SVR and non-SVR patients by Fisher's exact probability test. The longitudinal axis shows the –log *P* value. **b** Sequence alignment in the core region. *Dashes* indicate amino acids identical to the consensus sequence and substituted amino acids are shown by standard single letter codes. **c** Sliding window analysis. Viral regions affecting treatment outcomes are shown in *dark spots*. There are four hot spots: at core amino acid 110, amino acids 400–403 (i.e., the hypervariable region) in Envelope 2 (*E2*) region, amino acids 724–743 in E2, and amino acids 2258–2306 in the nonstructural (*NS*) 5A. **d** Sequence alignment amino acids in the nonstructural (*NS*) 5A around amino acids 2258–2306. *Dashes* indicate amino acids identical to the consensus sequence and substituted amino acids are shown by standard *single letter* codes

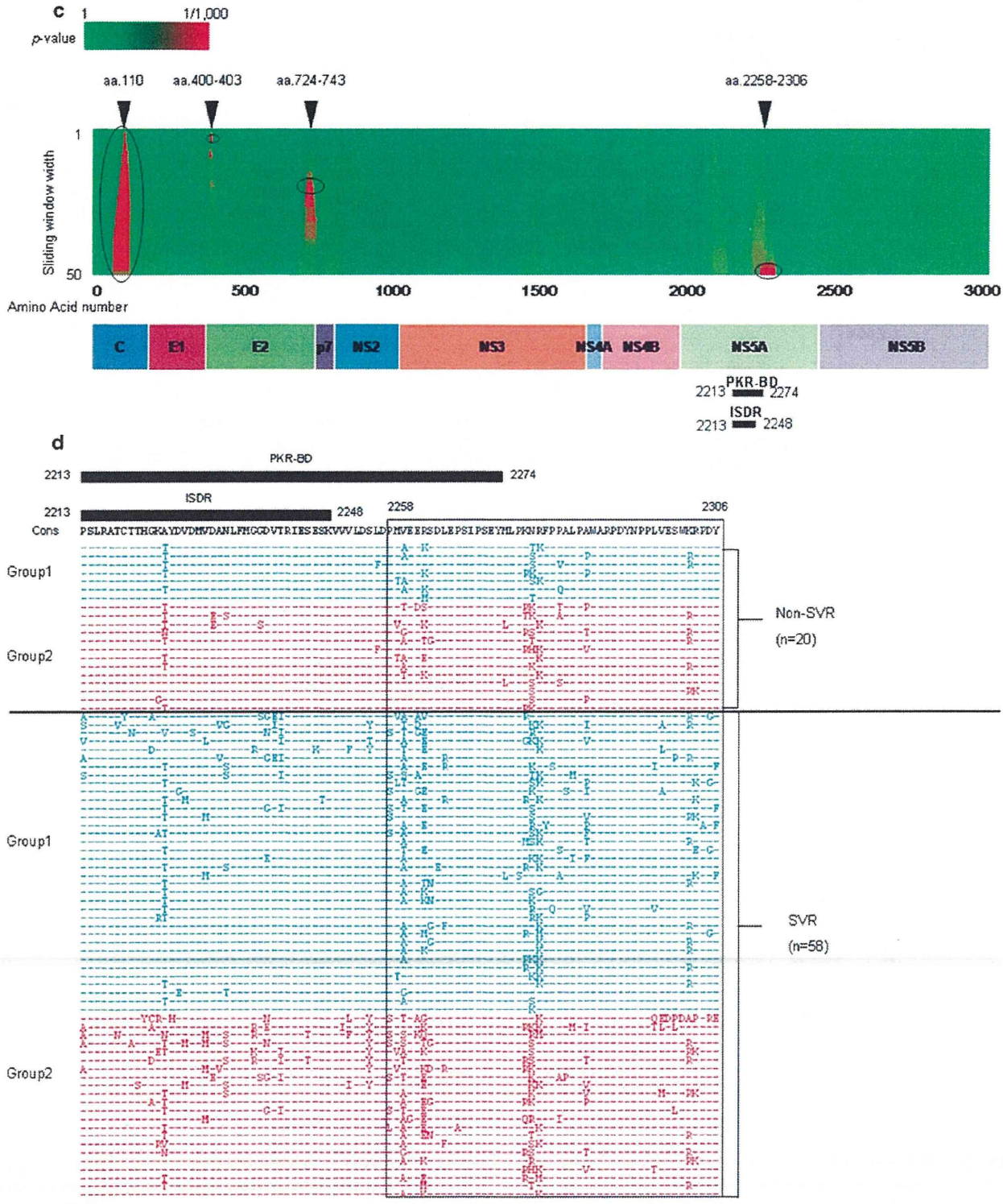

Fig. 2 continued

non-T (N/S) to T were significantly more frequent in SVR (32/58, 55.2%) than in non-SVR (1/20, 3.6%, $p = 5E{-}05$). Amino acid 4, the site reported recently to vary according to the viral response in genotype 2a infection, did not differ significantly in our study. Amino acid 70 and 91, which have been reported to vary according to viral response to

Table 2 Variations in each amino acid position and SVR rate

Position	Group 1 ($n = 43$)	p value	Group 2 ($n = 35$)	p value	Combined ($n = 78$)	p value
Core aa 110						
T	100% (19/19)	0.01	92.9% (13/14)	0.004	97% (32/33)	5E−05
Non T	70.8% (17/24)		42.9% (9/21)		57.8% (26/45)	
p7 aa 773						
V	77.4% (24/31)	0.16	53.6% (15/28)	0.03	66.1% (39/59)	0.002
Non V	100% (12/12)		100% (7/7)		100% (19/19)	
NS5A aa 2099						
R	92.9% (13/14)	0.40	91.7% (11/12)	0.01	92.3% (24/26)	0.01
Non R	79.3% (23/29)		47.8% (11/23)		65.4% (34/52)	
NS5B aa 3013						
L	78.9% (26/33)	0.17	47.8% (11/23)	0.01	66.1% (37/56)	0.008
Non L	100% (10/10)		91.7% (11/12)		95.5% (21/22)	

Table 3 Number of amino acid substitutions in each region and SVR rate

Region	Group 1 ($n = 43$)	p value	Group 2 ($n = 35$)	p value	Combined ($n = 78$)	p value
E2 aa 400–403						
Mutation ≥ 2	89.3% (25/28)	0.22	100% (11/11)	0.002	92.3% (36/39)	0.0005
Mutation 0–1	73.3% (11/15)		45.8% (11/24)		56.4% (22/39)	
E2 aa 724–743						
Mutation ≥ 1	100% (28/28)	0.0002	72% (18/25)	0.12	86.8% (46/53)	0.0006
No mutation	53.3% (8/15)		40% (4/10)		48% (12/25)	
ISDR(aa 2213–2248)						
Mutation ≥ 2	100% (15/15)	0.08	86.7% (13/15)	0.02	93.3% (28/30)	0.003
Mutation 0–1	75% (21/28)		45% (9/20)		62.5% (30/48)	
NS5A aa 2258–2306						
Mutation ≥ 5	100% (19/19)	0.01	84.2% (16/19)	0.006	92.1% (35/38)	0.0006
Mutation 0–4	70.8% (17/24)		37.5% (6/16)		57.5% (23/40)	

PEG-IFN/RBV therapy in genotype 1b infection, were conserved irrespective of the outcome.

Comparison of amino acid variation between the SVR and non-SVR patients across HCV "regions" using sliding window analysis

Figure 2c shows the combined result of sliding window analysis for study groups 1 and 2. This approach was used to detect differing HCV amino acid "regions", rather than single amino acid positions, between the SVR and the non-SVR patients. According to the result, four regions were notably associated with the final outcome (p values less than 1/1,000). Core aa 110, detected as a single amino acid position discriminating between the SVR and the non-SVR patients, was also identified as one of these regions. Because core aa 110 was already known for its strong correlation with the response as above, the region was excluded from further analysis. Among the other three regions, only NS5A aa 2258–2306 showed significant differences in the two independent study groups (Table 3). Interestingly, the NS5A region overlapped the PKR-binding domain, which includes the IFN sensitivity determining region (ISDR). Figure 2d shows the aligned sequences of amino acids around 2258–2306 of HCV NS5A. As with previous studies, variations in the ISDR were also significantly more frequent in SVR patients.

Multivariate analysis to detect independent factors contributing to the SVR

Multivariate analysis revealed that variation of core aa 110, the total number of substitutions within NS5A aa 2258–2306, and total RBV dose $\geq$80% were finally

Table 4 Multivariate logistic regression analysis

Factor	Odds (95% CI)	*p* value
Age	1.01 (0.91–1.13)	0.85
HCV RNA	1.00 (1.00–1.00)	0.09
Fibrosis score ≥3/0–2	2.37 (0.21–26.7)	0.48
RVR achievement	3.46 (0.54–22.1)	0.19
Ribavirin dose ≥80%	16.0 (1.66–153)	0.02
Core aa 110 T	24.7 (1.72–353)	0.02
NS5A aa 2258–2306 mutations 0–4/≥5	11.5 (1.23–108)	0.03

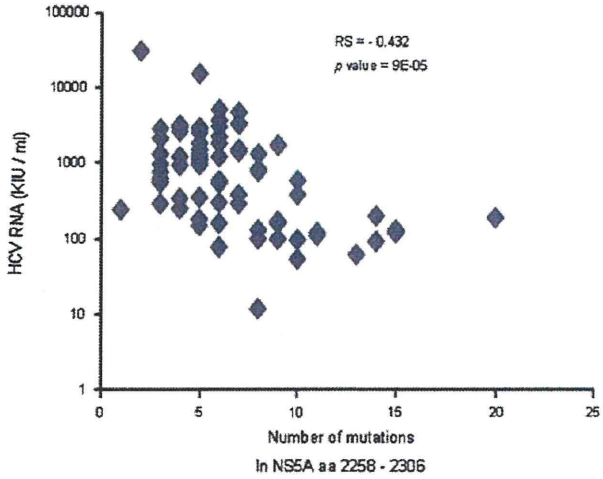

Fig. 3 Correlation between pretreatment HCV RNA levels and the number of substitutions in the NS5A region aa 2258–2306. Spearman's correlation coefficient by rank test is demonstrated

identified as the independent variables influencing the final outcome (odds ratio 24.7, 11.5, and 16.0; $p = 0.02$, 0.03 and 0.02; Table 4).

Biological relevance of variation in core and NS5A in this study group

To determine biological relevance of core aa110 and NS5A aa2258–2306, we investigated their relationship with clinical background factors. Multiple variations in the NS5A region aa 2258–2306 were significantly related to pretreatment HCV RNA titer ($p = 9E{-}05$, Fig. 3; Table 5). Interestingly, variation of the core aa110 was significantly associated with the patients' age ($p = 0.03$, Table 6).

Discussion

In this study, based on analysis of complete HCV-ORF sequences and comparison of SVR and non-SVR patients in two independent study groups, we have shown that amino acid variations in the core and NS5A correlate most significantly with the final outcome in the treatment for genotype 2a chronic hepatitis C. The study is unique in that the patients studied were all Japanese, excluding any effect of racial differences and providing a clearer analysis of the viral differences.

From the analysis of the characteristics of patients infected with genotype 2a HCV, it was clear that most non-SVR patients responded to the PEG-IFN/RBV therapy at least transiently, given that most of these non-SVR patients (89%) achieved EVR. This result demonstrated that most non-SVR patients were relapsers, but were not null-responders as observed frequently among genotype 1b patients treated with PEG-IFN/RBV therapy. Therefore, we compared the different viral responses according to the final outcome of SVR or non-SVR.

Variation of core aa 110 was identified as the single amino acid residue most significantly related to the final outcome ($p = 5E{-}05$). In recent studies of treatment of genotype 1b infection with PEG-IFN/RBV, amino acid variation in the core region was reported to be associated with response. It is interesting that the core region was also identified as a HCV gene associated with the response to PEG-IFN/RBV therapy of genotype 2a infection, although the amino acid residues of core in genotype 1b were different, being aa 70 and aa 91. It is also interesting that amino acids aa 70 and aa 91 are conserved as arginine and leucine, respectively, in genotype 2a, as reported to be associated with favorable PEG-IFN/RBV responses in genotype 1b infection, consistent with the association with a high SVR rate in genotype 2a infection. Very recently, a correlation was reported between amino acid variations in the core region and viral responses of genotype 2a HCV infection [20]. Though the result seems discrepant from our study, we suspect the inconsistent results were at least partially attributable to the different groups used in comparison: we compared the difference between non-SVR patients and SVR patients while they compared the difference between non-SVR and RVR patients.

In systemic searching for the viral "regions" associated with the treatment outcome, NS5A aa2258–2306 was identified by two independent studies. Interestingly, the region overlaps the PKR-binding domain (PKR-BD), including the ISDR, in which the number of amino acid substitutions is known to be related to the response to IFN-based therapy in genotype 1b, and also in genotype 2a [17, 18]. Therefore, we also confirmed that total number of substitutions in the ISDR and PKR-BD is significantly associated with the final outcome in this group of patients when the two studies were combined.

Some viral regions other than core and NS5A also showed the potential association with the final outcome. Viral single amino acid substitutions of aa 773 in p7, aa

Table 5 Baseline characteristics of patients with NS5A aa 2258–2306 mutations 0–4 or ≥5 (groups 1 and 2)

Characteristic	Mutation 0–4 ($n = 40$)	Mutation ≥ 5 ($n = 38$)	p value
Gender (male/female)	22/18	16/22	NS†
Age (years)	54.3 ± 11.4*	53.5 ± 11.5	NS‡
ALT (IU/l)	73.8 ± 70.3	85.3 ± 78.7	NS‡
Platelet ($\times 10^4/mm^3$)	18.0 ± 5.9	21.0 ± 5.7	0.03‡
Fibrosis score (0–2/≥3)§	33/5	33/2	NS†
HCV RNA (KIU/ml)	1,100 (99–30,000)**	380 (12–5,000)	0.02‖
IFN dose (≥80%/60–80%)¶	31/8	33/2	NS†
Ribavirin dose (≥80%/60–80%)¶	25/14	28/7	NS†
RVR rate (%)	65.8	62.9	NS†
EVR rate (%)	94.7	100	NS†
Relapse rate (%)	35.9	7.9	0.002†
SVR rate (%)	57.5	92.1	0.0006†

* Mean ± SD; † Fisher's exact probability test; ‡ Student t test; § mutation 0–4 $n = 38$, mutation ≥5: $n = 35$; ** median (range); ‖ Mann–Whitney's U test; ¶ mutation 0–4: $n = 39$, mutation ≥5: $n = 35$

Table 6 Baseline characteristics of patients with core 110 T or N/S (groups 1 and 2)

Characteristic	Core 110 T ($n = 33$)	Core 110 N/S ($n = 45$)	p value
Gender (male/female)	18/15	20/25	NS†
Age (years)	50.4 ± 13.0*	56.4 ± 9.5	0.032‡
ALT (IU/l)	64.5 ± 48.2	88.8 ± 86.2	NS‡
Platelet ($\times 10^4/mm^3$)	19.3 ± 4.9	19.5 ± 6.6	NS‡
Fibrosis score (0–2/≥3)§	30/1	36/6	NS†
HCV RNA (KIU/ml)	580 (54–3,600)**	980 (12–30,000)	NS‖
IFN dose (≥80%/60–80%)¶	26/3	38/7	NS†
Ribavirin dose (≥80%/60–80%)¶	23/6	30/15	NS†
RVR rate (%)	72.4	59.1	NS†
EVR rate (%)	100	95.5	NS†
Relapse rate (%)	3.0	38.6	9E−05†
SVR rate (%)	97.0	57.8	5E−05†

* Mean ± SD; † Fisher's exact probability test; ‡ Student t test; § core 110 T: $n = 31$, core 110 N/S: $n = 42$; ** median (range), ‖ Mann–Whitney's U test; ¶ core 110 T: $n = 29$

2099 in the NS5A, and aa 3013 in NS5B, or viral regions in E1 aa 400–403 and in E2 aa 724–744 were more frequent in SVR. However, because these were not extracted as significant in one of the two studies when analyzed separately, additional studies are needed to confirm the association with the final outcome. On the other hand, we could not find an association with the final outcome and the PePHD or IRRDR, including the V3 regions (data not shown) reported 1b HCV infection [21, 22].

It is interesting that the variation of the core region showed clear association with age. Younger patients with core aa 110T showed favorable responses, while older patients with core aa 110 non-T showed unfavorable responses. It is possible that different response rates according to the patients' ages in genotype 2a infection might have been related to the core substitutions, although further study is needed. In NS5A, it was reported that the variations within the PKR-binding region, including those within the ISDR, can disrupt the NS5A-PKR interaction, possibly rendering HCV sensitive to the antiviral effects of IFN [23]. Clinically, the number of substitutions within the region has been reported to correlate with the serum HCV RNA level [12]. We also confirmed that the number of substitutions within the NS5A aa 2258–2306 was significantly associated with the pretreatment HCV RNA titers.

Multivariate analysis of the combined group of patients showed that variation of core aa 110, NS5A aa 2258–2306, and total RBV dose ≥80% were independent variables associated with the final outcome (Table 4). The association of RBV dose and HCV relapse rate was reported previously [24] and that result was confirmed in this study. On the other hand, the total PEG-IFN dosage was not identified when it was administered at greater than 60% of the initially scheduled amount. Indeed, when the drug dosage was excluded, the strongest association was seen in the viral elements of core and NS5A, revealing the

importance of these two regions in the treatment of genotype 2a HCV infection with PEG-IFN/RBV therapy.

On the other hand, our study still has some limitations. In recent studies, IL28B single nucleotide polymorphisms were reported to be correlated significantly with the treatment response in genotype 1b HCV infections [25, 26]. In genotype 2a HCV infection, a correlation was also reported to exist between the IL28B SNP and the treatment response [27]. However, we could not investigate the association of the IL28B single nucleotide polymorphisms in the treatment response in genotype 2a HCV infections. In addition, the number of analyzed patients was rather small, especially in non-SVR patients.

In conclusion, by comprehensive investigation of the complete HCV ORF in patients showing different responses to PEG-IFN/RBV therapy, we have demonstrated that amino acid variation in the core and NS5A are significantly associated with the final outcome of treatment of genotype 2a chronic hepatitis C. Considering this result, determination of those HCV regions before treatment might provide further benefits for the patients infected with genotype 2a HCV.

Acknowledgements This study was supported in part by a grant-in-aid scientific research fund of the Ministry of Education, Science, Sports and Culture number 20390206 and in part by a grant-in-aid from the Ministry of Health, Labour, and Welfare of Japan (H19-kanen-002). We are grateful to Yamanashi Pegintron Ribavirin Study Group and Ochanomizu Liver Conference Group for their cooperation and advices. Especially, we thank Asuka Kanayama and Takako Ohmori for their technical assistance, and Takatoshi Kitamura and Shunichi Okada for their cooperation and advices.

References

1. Ghany MG, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: an update. Hepatology 2009;49(4):1335–1374
2. Di Bisceglie AM, Martin P, Kassianides C, Lisker-Melman M, Murray L, Waggoner J, Goodman Z, Banks SM, Hoofnagle JH. Recombinant interferon alfa therapy for chronic hepatitis C A randomized, double-blind, placebo-controlled trial. N Engl J Med 1989;321(22):1506–1510
3. Haydon GH, Jarvis LM, Blair CS, Simmonds P, Harrison DJ, Simpson KJ, Hayes PC. Clinical significance of intrahepatic hepatitis C virus levels in patients with chronic HCV infection. Gut 1998;42(4):570–575
4. Simmonds P. Clinical relevance of hepatitis C virus genotypes. Gut 1997;40(3):291–293
5. Hadziyannis SJ, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H Jr, Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med 2004;140(5):346–355
6. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 2001;358(9286):958–965
7. Dalgard O, Bjoro K, Hellum KB, Myrvang B, Ritland S, Skaug K, Raknerud N, Bell H. Treatment with pegylated interferon and ribavarin in HCV infection with genotype 2 or 3 for 14 weeks: a pilot study. Hepatology 2004;40(6):1260–1265
8. Mangia A, Santoro R, Minerva N, Ricci GL, Carretta V, Persico M, Vinelli F, Scotto G, Bacca D, Annese M, Romano M, Zechini F, Sogari F, Spirito F, Andriulli A. Peginterferon alfa-2b and ribavirin for 12 vs 24 weeks in HCV genotype 2 or 3. N Engl J Med 2005;352(25):2609–2617
9. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 1996;334(2):77–81
10. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology 2008;48(1):38–47
11. Hamano K, Sakamoto N, Enomoto N, Izumi N, Asahina Y, Kurosaki M, Ueda E, Tanabe Y, Maekawa S, Itakura J, Watanabe H, Kakinuma S, Watanabe M. Mutations in the NS5B region of the hepatitis C virus genome correlate with clinical outcomes of interferon-alpha plus ribavirin combination therapy. J Gastroenterol Hepatol 2005;20(9):1401–1409
12. Chayama K, Suzuki F, Tsubota A, Kobayashi M, Arase Y, Saitoh S, Suzuki Y, Murashima N, Ikeda K, Takahashi N, Kinoshita M, Kumada H. Association of amino acid sequence in the PKR-eIF2 phosphorylation homology domain and response to interferon therapy. Hepatology 2000;32(5):1138–1144
13. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, Matsuda M, Arase Y, Ikeda K, Kumada H. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon–ribavirin combination therapy. Intervirology 2005;48(6):372–380
14. Toyoda H, Kumada T, Tada T, Arakawa T, Hayashi K, Honda T, Katano Y, Goto H. Association between HCV amino acid substitutions and outcome of peginterferon and ribavirin combination therapy in HCV genotype 1b and high viral load. J Gastroenterol Hepatol 2010;25(6):1072–1078
15. Donlin MJ, Cannon NA, Aurora R, Li J, Wahed AS, Di Bisceglie AM, Tavis JE. Contribution of genome-wide HCV genetic differences to outcome of interferon-based therapy in Caucasian American and African American patients. PLoS One 2010;5(2): e9032
16. Donlin MJ, Cannon NA, Yao E, Li J, Wahed A, Taylor MW, Belle SH, Di Bisceglie AM, Aurora R, Tavis JE. Pretreatment sequence diversity differences in the full-length hepatitis C virus open reading frame correlate with early response to therapy. J Virol 2007;81(15):8211–8224
17. Murakami T, Enomoto N, Kurosaki M, Izumi N, Marumo F, Sato C. Mutations in nonstructural protein 5A gene and response to interferon in hepatitis C virus genotype 2 infection. Hepatology 1999;30(4):1045–1053
18. Hayashi K, Katano Y, Honda T, Ishigami M, Itoh A, Hirooka Y, Nakano I, Urano F, Yoshioka K, Toyoda H, Kumada T, Goto H. Mutations in the interferon sensitivity-determining region of hepatitis C virus genotype 2a correlate with response to pegylated-interferon-alpha 2a monotherapy. J Med Virol 2009;81(3): 459–466
19. Kobayashi M, Watanabe K, Ishigami M, Murase K, Ito H, Ukai K, Yano M, Takagi K, Hattori M, Kakumu S, Yoshioka K. Amino acid substitutions in the nonstructural region 5A of hepatitis C virus genotypes 2a and 2b and its relation to viral

load and response to interferon. Am J Gastroenterol 2002;97(4): 988–998
20. Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 2a high viral load and virological response to interferon–ribavirin combination therapy. Intervirology 2009;52(6):301–309
21. Saito T, Ito T, Ishiko H, Yonaha M, Morikawa K, Miyokawa A, Mitamura K. Sequence analysis of PePHD within HCV E2 region and correlation with resistance of interferon therapy in Japanese patients infected with HCV genotypes 2a and 2b. Am J Gastroenterol 2003;98(6):1377–1383
22. Watanabe H, Nagayama K, Enomoto N, Itakura J, Tanabe Y, Sato C, Izumi N, Watanabe M. Amino acid substitutions in PKR-eIF2 phosphorylation homology domain (PePHD) of hepatitis C virus E2 protein in genotype 2a/2b and 1b in Japan and interferon efficacy. Hepatol Res 2003;26(4):268–274
23. Gale M Jr, Blakely CM, Kwieciszewski B, Tan SL, Dossett M, Tang NM, Korth MJ, Polyak SJ, Gretch DR, Katze MG. Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: molecular mechanisms of kinase regulation. Mol Cell Biol 1998;18(9):5208–5218
24. Hiramatsu N, Oze T, Yakushijin T, Inoue Y, Igura T, Mochizuki K, Imanaka K, Kaneko A, Oshita M, Hagiwara H, Mita E, Nagase T, Ito T, Inui Y, Hijioka T, Katayama K, Tamura S, Yoshihara H, Imai Y, Kato M, Yoshida Y, Tatsumi T, Ohkawa K, Kiso S, Kanto T, Kasahara A, Takehara T, Hayashi N. Ribavirin dose reduction raises relapse rate dose-dependently in genotype 1 patients with hepatitis C responding to pegylated interferon alpha-2b plus ribavirin. J Viral Hepat 2009;16(8):586–594
25. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461(7262):399–401
26. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 2009;41(10):1105–1109
27. Rauch A, Kutalik Z, Descombes P, Cai T, di Iulio J, Mueller T, Bochud M, Battegay M, Bernasconi E, Borovicka J, Colombo S, Cerny A, Dufour JF, Furrer H, Gunthard HF, Heim M, Hirschel B, Malinverni R, Moradpour D, Mullhaupt B, Witteck A, Beckmann JS, Berg T, Bergmann S, Negro F, Telenti A, Bochud PY. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure—a genome-wide association study. Gastroenterology 2010 Jan 7

Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in *IL28B* and viral factors

Masayuki Kurosaki[1], Yasuhito Tanaka[2], Nao Nishida[3], Naoya Sakamoto[4], Nobuyuki Enomoto[5], Masao Honda[6], Masaya Sugiyama[2], Kentaro Matsuura[2], Fuminaka Sugauchi[2], Yasuhiro Asahina[1], Mina Nakagawa[4], Mamoru Watanabe[4], Minoru Sakamoto[5], Shinya Maekawa[5], Akito Sakai[6], Shuichi Kaneko[6], Kiyoaki Ito[7], Naohiko Masaki[7], Katsushi Tokunaga[3], Namiki Izumi[1,*], Masashi Mizokami[2,7]

[1]*Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, Tokyo, Japan;* [2]*Department of Virology, Liver Unit, Nagoya City University, Graduate School of Medical Sciences, Nagoya, Japan;* [3]*Department of Human Genetics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan;* [4]*Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo, Japan;* [5]*First Department of Internal Medicine, University of Yamanashi, Yamanashi, Japan;* [6]*Department of Gastroenterology, Kanazawa University, Graduate School of Medicine, Kanazawa, Japan;* [7]*Research Center for Hepatitis and Immunology, International Medical Center of Japan, Konodai Hospital, Ichikawa, Japan*

Background & Aims: Pegylated interferon and ribavirin (PEG-IFN/RBV) therapy for chronic hepatitis C virus (HCV) genotype 1 infection is effective in 50% of patients. Recent studies revealed an association between the *IL28B* genotype and treatment response. We aimed to develop a model for the pre-treatment prediction of response using host and viral factors.

Methods: Data were collected from 496 patients with HCV genotype 1 treated with PEG-IFN/RBV at five hospitals and universities in Japan. *IL28B* genotype and mutations in the core and IFN sensitivity determining region (ISDR) of HCV were analyzed to predict response to therapy. The decision model was generated by data mining analysis.

Results: The *IL28B* polymorphism correlated with early virological response and predicted null virological response (NVR) (odds ratio = 20.83, $p < 0.0001$) and sustained virological response (SVR) (odds ratio = 7.41, $p < 0.0001$) independent of other covariates. Mutations in the ISDR predicted relapse and SVR independent of *IL28B*. The decision model revealed that patients with the minor *IL28B* allele and low platelet counts had the highest NVR (84%) and lowest SVR (7%), whereas those with the major *IL28B* allele and mutations in the ISDR or high platelet counts had the lowest NVR (0–17%) and highest SVR (61–90%). The model had high reproducibility and predicted SVR with 78% specificity and 70% sensitivity.

Conclusions: The *IL28B* polymorphism and mutations in the ISDR of HCV were significant pre-treatment predictors of response to PEG-IFN/RBV. The decision model, including these host and viral factors may support selection of optimum treatment strategy for individual patients.

Keywords: *IL28B*; ISDR; Peg-interferon; Ribavirin; Data mining; Decision tree.
Received 14 March 2010; received in revised form 22 June 2010; accepted 7 July 2010
* Corresponding author. Address: Division of Gastroenterology and Hepatology, Musashino Red Cross Hospital, 1-26-1 Kyonan-cho, Musashino-shi, Tokyo 180-8610, Japan. Tel.: +81 422 32 3111; fax: +81 422 32 9551.
E-mail address: nizumi@musashino.jrc.or.jp (N. Izumi).

Introduction

Hepatitis C virus (HCV) infection is the leading cause of cirrhosis and hepatocellular carcinoma worldwide [1]. The successful eradication of HCV, defined as a sustained virological response (SVR), is associated with a reduced risk of developing hepatocellular carcinoma. Currently, pegylated interferon (PEG-IFN) plus ribavirin (RBV) is the most effective standard of care for chronic hepatitis C but the rate of SVR is around 50% in patients with HCV genotype 1 [2,3], the most common genotype in Japan, Europe, the United States, and many other countries. Moreover, 20–30% of patients with HCV genotype 1 have a null virological response (NVR) to PEG-IFN/RBV therapy [4]. The most reliable method for predicting the response is to monitor the early decline of serum HCV-RNA levels during treatment [5] but there is no established method for prediction before treatment. Because PEG-IFN/RBV therapy is costly and often accompanied by adverse effects such as flu-like symptoms, depression and hematological abnormalities, pre-treatment predictions of those patients who are unlikely to benefit from this regimen enables ineffective treatment to be avoided.

Recently, it has been reported through a genome-wide association study (GWAS) of patients with genotype 1 HCV that single nucleotide polymorphisms (SNPs) located near the *IL28B* gene are strongly associated with a response to PEG-IFN/RBV therapy in

Please cite this article in press as: Kurosaki M et al. Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in *IL28B* and viral factors. J Hepatol (2010), doi:10.1016/j.jhep.2010.07.037

Research Article

Table 1. Baseline characteristics of all patients, and patients assigned to the model building or validation groups.

	All patients n = 496	Model group n = 331	Validation group n = 165
Gender: male	250 (50%)	170 (51%)	80 (48%)
Age (years)	57.1 ± 9.9	56.8 ± 9.7	57.5 ± 10.2
ALT (IU/L)	78.6 ± 60.8	78.1 ± 61.4	79.7 ± 59.6
GGT (IU/L)	59.3 ± 63.6	58.9 ± 62.0	60.2 ± 66.9
Platelets (10^9/L)	154 ± 53	153 ± 52	154 ± 56
Fibrosis: F3-4	121 (24%)	80 (24%)	41 (25%)
HCV-RNA: >600,000 IU/ml	409 (82%)	273 (82%)	136 (82%)
ISDR mutation: ≤1	220 (88%)	290 (88%)	145 (88%)
Core 70 (Arg/Gln or His)	293 (59%)/203 (41%)	197 (60%)/134 (40%)	96 (58%)/69 (42%)
Core 91 (Leu/Met)	299 (60%)/197 (40%)	200 (60%)/131 (40%)	99 (60%)/66 (40%)
IL28B: Minor allele	151 (30%)	101 (31%)	50 (30%)
SVR	194 (39%)	129 (39%)	65 (39%)
Relapse	152 (31%)	103 (31%)	49 (30%)
NVR	150 (30%)	99 (30%)	51 (31%)

ALT, alanine aminotransferase; GGT, gamma-glutamyltransferase; ISDR, interferon sensitivity determining region; Arg, arginine; Gln, glutamine; His, histidine; Leu, leucine; Met, methionine; Minor, heterozygote or homozygote of minor allele; SVR, sustained virological response; NVR, null virological response.

Japanese [6], European [7], and a multi-ethnic population [8,9]. The last three studies focused on the association of SNPs in the *IL28B* region with SVR [7–9] but we found a stronger association with NVR [6]. In addition to these host genetic factors, we have reported that mutations within a stretch of 40 amino acids in the NS5A region of HCV, designated as the IFN sensitivity determining region (ISDR), are closely associated with the virological response to IFN therapy: a lower number of mutations is associated with treatment failure [10–13]. Amino acid substitutions at positions 70 and 91 of the HCV core region (Core70, Core91) also have been reported to be associated with response to PEG-IFN/RBV therapy: glutamine (Gln) or histidine (His) at Core70 and methionine (Met) at Core91 are associated with treatment resistance [4,14]. The importance of substitutions in the HCV core and ISDR was confirmed recently by a Japanese multicenter study [15]. How these viral factors contribute to response to therapy is yet to be determined. For general application in clinical practice, host genetic factors and viral factors should be considered together.

Data mining analysis is a family of non-parametric regression methods for predictive modeling. Software is used to automatically explore the data to search for optimal split variables and to build a decision tree structure [16]. The major advantage of decision tree analysis over logistic regression analysis is that the results of the analysis are presented in the form of flow chart, which can be interpreted intuitively and readily made available for use in clinical practice [17]. The decision tree analysis has been utilized to define prognostic factors in various diseases [18–25]. We have reported recently its usefulness for the prediction of an early virological response (undetectable HCV-RNA within 12 weeks of therapy) to PEG-IFN/RBV therapy in chronic hepatitis C [26].

This study aimed to define the pre-treatment prediction of response to PEG-IFN/RBV therapy through the integrated analysis of host factors, such as the *IL28B* genetic polymorphism and various clinical covariates, as well as viral factors, such as mutations in the HCV core and ISDR and serum HCV-RNA load. In addition, for the general application of these results in clinical practice, decision models for the pre-treatment prediction of response were determined by data mining analysis.

Materials and methods

Patients

This was a multicentre retrospective study supported by the Japanese Ministry of Health, Labor and Welfare. Data were collected from a total of 496 chronic hepatitis C patients who were treated with PEG-IFN alpha and RBV at five hospitals and universities throughout Japan. Of these, 98 patients also were included in the original GWAS analysis [6]. The inclusion criteria in this study were as follows (1) infection by genotype 1b, (2) lack of co-infection with hepatitis B virus or human immunodeficiency virus, (3) lack of other causes of liver disease, such as autoimmune hepatitis, and primary biliary cirrhosis, (4) completion of at least 24 weeks of therapy, (5) adherence of more than 80% to the planned dose of PEG-IFN and RBV for the NVR patients, (6) availability of DNA for the analysis of the genetic polymorphism of *IL28B*, and (7) availability of serum for the determination of mutations in the ISDR and substitutions of Core70 and Core91 of HCV. Patients received PEG-IFN alpha-2a (180 μg) or 2b (1.5 μg/kg) subcutaneously every week and were administered a weight adjusted dose of RBV (600 mg for <60 kg, 800 mg for 60–80 kg, and 1000 mg for >80 kg daily) which is the recommended dosage in Japan. Written informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committee. The baseline characteristics are listed in Table 1. For the data mining analysis, 67% of the patients (331 patients) were assigned randomly to the model building group and 33% (165 patients) to the validation group. There were no significant differences in the clinical backgrounds between these two groups.

Laboratory and histological tests

Blood samples were obtained before therapy and were analyzed for hematologic tests and for blood chemistry and HCV-RNA. Sequences of ISDR and the core region of HCV were determined by direct sequencing after amplification by reverse-transcription and polymerase chain reaction as reported previously [4,11]. Genetic polymorphism in one tagging SNP located near the *IL28B* gene (rs8099917) was determined by the GWAS or DigiTag2 assay [27]. Homozygosity (GG) or heterozygosity (TG) of the minor sequence was defined as having the *IL28B* minor allele, whereas homozygosity for the major sequence (TT) was

Please cite this article in press as: Kurosaki M et al. Pre-treatment prediction of response to pegylated-interferon plus ribavirin for chronic hepatitis C using genetic polymorphism in *IL28B* and viral factors. J Hepatol (2010), doi:10.1016/j.jhep.2010.07.037